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SAFETY AND IMMUNOGENICITY TESTING OF A PILOT POLYSACCHARIDE VAC--ETC(U)
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SAFETY AND IMMUNOGENICITY TESTING OF A PILOT POLYSACCHARIDE
VACCINE PREPARATION TO PSEUDOMONAS AERUGINOSA

ANNUAL REPORT

LEVEL II

Gerald B. Pier, Ph.D.

September 1979

(For period 1 March 1979 to 15 August 1979)

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Fort Detrick, Frederick, Maryland 21701

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Boston, Massachusetts 02115

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Purification techniques were developed for obtaining a high molecular weight polysaccharide preparation for testing as a vaccine to infections caused by the bacterium <u>Pseudomonas aeruginosa</u>. A pilot preparation from an immunotype 7 strain of this organism was made after consideration of using anion exchange resins and colonial morphology criteria for selecting techniques used for purification. Serological assays in the form of a radioactive antigen binding assay and a splenic plaque forming cell assay were developed to assay the polysaccharide preparations for immunogenicity. Finally testing of the polysaccharide prepara-		

20. Abstract (continued)

tions in animal models of P. aeruginosa infection have commenced.

Summary

The purpose of this work was to develop a safe and effective vaccine caused by the bacterium Pseudomonas aeruginosa. Methods were developed to isolate and purify this material from cultures of P. aeruginosa. The methods included an assessment of the effect of colonial morphology on polysaccharide production and the use of ion exchange resins for purification. Further methods included development of a radioactive antigen-binding assay and splenic plaque forming cell assay for assessment of the serological responses of immunized animals. The results indicate that ion exchange chromatography may be a useful purification method, and that careful attention to both colonial morphology and the pH during the acid hydrolysis step are critical for polysaccharide preparation. Other results include the successful demonstration of serologic activity in mouse and rabbit sera following immunization with polysaccharides. I conclude that further progress has been made towards the development of this vaccine.

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Foreword

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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A. Contract Background

The principal investigator was awarded a contract from the USAMRDC to run from 1 March 1979 to 28 February, 1980. The scope of this contract included preparation and testing of a pilot vaccine to Pseudomonas aeruginosa infections up to the human trial stage, development of assays for measuring the antibody response of immunized and infected individuals, testing of the immunogenicity of high molecular weight polysaccharides (PS) from P. aeruginosa in a plaque forming cell assay utilizing mouse spleen cells, and testing of PS preparations in other animal models such as a burned mouse. Around 1 July 1979 the principal investigator was requested to submit an annual report and renewal funding application as of 1 September, 1979 in order to bring the funding cycle of this contract into conformance with other similar contracts issued by the USAMRDC. Thus this annual report really represents a 5.5 month report. The principal investigator would like to further point out that since this is a brand new contract, the first two months were spent ordering and installing equipment, interviewing, hiring and training a new technician, and generally setting up and organizing the laboratory in order to accomplish the goals of this contract. Thus, real progress on the work proposed in this contract has only been going on for 3.5 months. In spite of the fact that substantial progress has been made in some of the areas, it is of necessity that this annual report is somewhat brief.

B. Progress on Vaccine Preparation

This contract proposed to prepare, by our previously described method (1) a high molecular weight polysaccharide (PS) preparation from immunotype 7 (IT-7) Pseudomonas aeruginosa, test this product for chemical composition, serological activity, immunogenicity and protective efficacy in mice, determine its molecular size, and test its toxicity in rabbits, mice, guinea pigs and monkeys. We were initially confronted in trial attempts at preparing an IT-7 PS vaccine preparation with good yields of a product which had very poor serological and mouse protective activity. Previous lots of IT-7 PS reacted in immunodiffusion tests with homologous whole organism antisera at a concentration of 0.5 mg/ml. The newer lots required 10 mg/ml in order to visualize a precipitin line. Furthermore, the newer lots only provided about 50 % protection in immunized mice, compared to 80-100% protection achieved with earlier lots. This lead us to speculate that the newer lots were possibly contaminated with other materials, so we next attempted separation on DEAE-Sephacel columns in 0.05 M Tris buffers. Utilizing 0-2M sodium chloride gradients to elute the column with, we found these newer, trial lots of IT-7 PS could be fractionated into as many as nine distinct peaks as measured by the absorbance of the column eluate at 206 nanometers (OD_{206}). Most of the material was recovered in the first 5 peaks eluting at NaCl molarities of between 0.03 and 1.0. Two to four more peaks were recovered in NaCl molarities of 1.0-2.0 M. Serological activity could only be recovered in the last eluting peak, having the highest salt molarity. After DEAE fractionation the various peaks were found to be chemically similar and only gave low levels of protection when used in mouse immunization-challenge studies. Thus no peak could be identified as the immunogenic one, and serological activity was only recovered in a fraction representing no more than 10% of the total material.

Further attempts to define the cause of the low serologic and immunogenic activity of these trial IT-7 PS preparations lead us to examine our stock bacterial strains. Although the strains appeared to be immunologically and biochemically stable, it was decided to open new lyophilized cultures of all the seven immunotypes of *P. aeruginosa* routinely employed. When these cultures were plated out on agar we noted that some cultures gave almost 100% colonies with a smooth, circular edge, whereas others gave mixtures of this colony type and a second colony type with the appearance of a "fried egg". IT-7 gave a mixture of types. Selection of these colony types for stable "smooth" and "fried-egg" appearances followed by small scale PS production from IT-7 "smooth" and "fried-egg" colonies indicated that the "smooth" colony gave serologically reactive PS whereas the "fried-egg" colony produced little to no PS. Thus the production in trial preparations of low reactivity vaccine appears to be due to the preponderance in unselected cultures of "fried-egg" colony types.

After these problems were worked out, the first vaccine lot, designated T-7 VL-1, was prepared starting on July 9, 1979. An accurate day by day log of all steps, reagents and procedures performed was kept. Two new procedures were introduced into preparing the vaccine lot. The first was the passage through PM-30 membranes of the growth media prior to autoclaving in order to eliminate from the media any high molecular weight media components that could possibly contaminate our vaccine. Alcohol precipitation of unfiltered media, (Tryptacase soy broth, TSB, with 3 % glycerol), followed by column chromatography showed the presence of a high molecular weight component eluting in the void volume of a Sepharose CL6B column, 1.6 x 60 cm. Analyses by gas liquid chromatography of this product did not reveal the presence of any sugar units, but it was decided to ultrafilter the media anyway in order to eliminate the possibility of its occurrence in the final vaccine product.

The second change came when it was noted that dissolution of crude material in 1% acetic acid, prior to the mild acid hydrolyses step employed to eliminate lipopolysaccharide (LPS) contamination, resulted in a pH of 7.5. Since the pH of 1% acetic acid is 2.8, two 5.0 ml aliquotes were simultaneously tested for the feasibility of LPS elimination at either 2.5% acetic acid (pH 5.2) or at pH 2.8 (50% acetic acid). It was found that after hydrolysis at pH 5.2, the 2.5% acetic acid aliquote contained precipitated lipid A, gave a clearly defined PS line in immunodiffusion gels, and contained no serologically or chemically detectable LPS. The pH 2.8 lot was unreactive in immunodiffusion gels, indicating that only acetate ion and heat are needed for *P. aeruginosa* LPS cleavage, and that low pH and high acetate ion concentrations destroy PS reactivity. Thus the remainder of T-7 VL-1 was prepared utilizing 2.5% acetic acid hydrolysis.

On 15 August, 1979 14.6 mg of T-7 VL-1 were obtained as a final product. The low yield obtained indicated a loss of unknown cause. Thus on 16 August 1979 preparation of a second lot of IT-7 PS, T-7 VL-2, was started. T-7 VL-1 gave good reactivity in serological assays (immunodiffusion and hemagglutination inhibition) and will be used to check the progress and reactivity of T-7 VL-2. Upon completion of the T-7 VL-2 preparation, the material will be bottled prior to safety and immunogenicity testing at the Massachusetts State Laboratory, Boston, Massachusetts.

C. Progress of Development of Serological Assays for Antibody Response

1). Radioactive antigen binding test.

Extrinsic labelling of PS with ^3H by the method of Keck was worked out for IT-1 thru IT-7 PS. High titered rabbit antisera prepared to P. aeruginosa organisms was then used to quantitate the binding to labelled PS by correlation with quantitative precipitin curves. Table 1 shows the specific activity of IT-1 - IT-7 ^3H -PS, equivalence points obtained for each antisera, the percent of the counts bound by each antisera at an antigen input of 250 ng, and the calculated point of 100% binding utilizing 50 ul of antisera. It can be seen that all of these sera bound 100% of the antigen at 30-80 ng input for 50 ul of sera. Since the specific activities of the ^3H -PS were 0.5-2.5 (CPM/ng), the low amount of antigen needed for 100% binding was not compatible with the specific activities obtained in order to utilize this RABA. Three approaches are currently under consideration to remedy this problem.

a). Use of a "hotter" labelling agent, Na BH_4^3 with a higher specific activity of counts per millimole will be tried in order to make a "hotter" antigen.

b). P. aeruginosa grow well in media consisting only of phosphate, magnesium, ammonia, sulfate and a carbon source. Currently we are preparing PS antigens testing four different carbon sources: citrate, acetate, glucose, and glycerol. All of these carbon sources are available as C^{14} or H^3 labelled compounds, and if serologically active and chemically complete PS can be isolated from one of these minimal media, the appropriately labelled PS will be prepared.

c). If neither of the above is feasible, the PS can be labelled with I^{125} by the method of Keck (2), introducing tyramine groups instead of ^3H to the periodate treated PS. This method has been tried previously and found successful, but was abandoned in favor of working with ^3H .

2). Enzyme linked immunoadsorbant assay (ELISA). No work has been done on this aspect yet but is planned for later this year.

D. Progress of Development of Plaque Forming Cell (PFC) Test for Immunogenicity of PS in Mice.

This assay has been very successful for us in the past 4 months and we have generated a fair amount of data in this time regarding the immunologic behavior of mice towards PS. We have found that $\text{C}_3\text{H}/\text{ANF}$ mice respond to IT-1 PS in a typical dose - response fashion, generating a maximal response of 2854 IqM plaques/spleen at a 1 ug/mouse dose (Table 2). This correlates perfectly with the protection seen upon challenge of these mice with live, IT-1 organisms. Table 3 shows that at a 1 ug/mouse dose of IT-1 PS, 80% of $\text{C}_3\text{H}/\text{ANF}$ mice are protected. Mice immunized with lower or higher doses are not protected to a significant degree. Finally 0.2 ml of sera from $\text{C}_3\text{H}/\text{ANF}$ mice immunized with 1 ug of IT-1 PS passively transfers protection to Balb/C mice, whereas 0.3 ml of serum from Balb/C mice immunized with 50 ug of IT-1 PS is non-protective (Table 4).

Balb/C mice were found to be non-responders to IT-1 PS in terms of generation of a PFC response over a wide dosage range. However, Table 5 shows that Balb/C mice were protected to challenge with low doses of live organisms (1 LD_{100}) at doses of 50 ug/mouse. This is the same dosage level we have found to be

protective in outbred mice. Thus there appears to be two separate mechanisms of protection generated by PS immunization. The first is represented by the C₃H/ANF which give a clear antibody response in a typical dose response fashion, and transfer of serum from these immunized mice passively confers protection. The second is represented by the Balb/C and outbred mice wherein higher doses are needed to confer protection, no PFC antibody response is measurable, and passive transfer of serum from immunized animals is non-protective. Currently we are studying the mechanism of protection in the Balb/C mice. Transfer of spleen cells and higher amounts of serum are being tried. Other studies in regards to immunological control of the response to IT-1 PS include kinetics of the response, generation and transfer of suppression of the response, T-cell dependence or independence of the response, genetics of the response, and the relation of PS to the immunologically similar LPS responses. The latter is important in regards to ascertaining if LPS contamination of PS preparations is responsible for protective immunity. Preliminary data indicate that at high doses of LPS (100 ug/mouse) a PFC response comparable to that seen with 1.0 ug of PS is elicited, suggesting that LPS does not follow the same dose-response curve that PS does. Finally, since mice with a C₃H genetic background are responders to PS, and a strain of this mouse, C₃H/HeJ is a known non-responder to LPS (3), P. aeruginosa PS and LPS which share immunologic determinants may prove to be valuable tools in studying the genetic and biochemical basis of LPS responses in these mouse strains.

E. Progress on Expanding the Animal Model

The contract calls for testing of PS vaccine efficacy in burned mouse and wounded rat models. Dr. I.A. Holder of the Shriners Burns Institute, Cincinnati, Ohio has been provided with PS prepared from strain M-2 of P. aeruginosa. Dr. Holder is currently preparing to test this PS in a burned mouse model (4). Dr. A. McManus of the U.S. Army Institute of Surgical Research, Ft. Sam Houston, Houston, Texas was not able to demonstrate any protective efficacy of PS vaccination in wounded rats. However, Dr. McManus reports that he only obtains protection in animals immunized with a sub-lethal dose of live organisms, calling into question the appropriateness of this model.

In addition to these two tests, Dr. J. Pennington of Harvard Medical School has been testing the efficacy of IT-1 PS in protecting guinea pigs against P. aeruginosa acute hemorrhagic pneumonia. His model (5) involves direct tracheal installation of organisms into the lungs. His results have been encouraging. In 4 separate experiments, five of eight immunized animals survived challenge with varying doses of IT-1 P. aeruginosa. Some problems in the virulence of the IT-1 strain provided seem to have hampered some experiments, but we believe this could be traced to the "smooth" versus "fried-egg" morphology seen in cultures of P. aeruginosa. The interesting aspect of Dr. Pennington's work has been that the guinea pigs seem to respond to IT-1 PS immunization in a manner analogous to the Balb/C mice. Thus they require high doses of PS for immunization and we cannot demonstrate antibody rises in an assay like hemagglutination. It is likely that the antibody produced can be measured in either the RABA or the ELISA. Since it is far easier to obtain quantities of sera from guinea pigs than mice, Dr. Pennington's work will be of immense value in defining the nature of protective immunity generated by PS immunization.

Table 1. Properties of Extrinsically Labelled PS Antigens¹

Antigen from Immunotype	Specific Activity	Equivalence Point of 1 ml sera	Percent CPM Bound 250 ng input	100% Binding Point
1	2.50 ²	1338 ³	83.1 ⁴	80 ⁵
2	0.99	340	47.0	50
3	1.02	3040	38.7	62.5
4	0.62	220	32.9	30
5	0.52	1220	50.7	62.5
6	1.81	220	29.2	NM ⁶
7	1.10	1920	36.1	NM ⁶

1 Antigens labelled with H³ by method of Keck (2)

2 Counts per minute/nanogram

3 Ug of Ps antigen bound/ml of antisera to whole organisms

4 Percentage of input counts bound when antigen input is 250 ng

5 Amt of antigen (ng) where 100% is bound by 50 ul of antisera to whole organisms

6 NM - not measurable

Table 2. Plaque forming Cell Response of C₃H/ANF mice to Immunization with IT-1 PS: Response at Day 5.

Immunizing Dose (ug)	IgM Plaques Per Spleen ¹	IgG Plaques Per Spleen
0.1	169	107
0.5	1271	286
1.0	2854	1293
2.0	1718	1539
5.0	1508	1781
10.0	646	745
50.0	7.3	7.0

¹ Average of 5 mice per point.

Table 3. Survival of C₃H/ANF Mice Immunized with IT-1 PS

Immunizing Dose (ug)	<u>No. Alive</u> Total (per cent survivors)		Challenge Dose	No. LD ₁₀₀
50	0/5	(0)	1.3×10^8	5.2
5	0/5	(0)	"	"
1	4/5	(80)	"	"
0.1	1/5	(20)	"	"
0	0/5	(0)	"	"
<hr/>				
50	5/5	100	2.5×10^7	1.0
5	5/5	100	"	"
1	5/5	100	"	"
0.1	5/5	100	"	"
0	0/5	0	"	"
<hr/>				

Mice given indicated amount of antigen in 0.1 ml saline followed on day 7 with challenge by live IT-1 P. aeruginosa in 0.1 ml saline.

Table 4. Passive Transfer of Serum from C₃H/ANF and Balb/C Mice in Protection of Balb/C Mice from Challenge

Serum Given ¹	Amt (ml)	No. Alive Total	% Survivors	Challenge Dose
C ₃ H/ANF ²	0.2	4/5	80	1.2 x 10 ⁸
Balb/C ³	0.3	1/5	20	"
None	--	0/5	0	"

1 Mice given serum 1 hr. prior to challenge with 0.1 ml of live IT-1 P. aeruginosa

2 C₃H/ANF serum obtained from mice given 1.0 ug. of IT-1 Ps.

3 Balb/C serum obtained from mice given 50.0 ug. of IT-1 Ps.

Table 5. Survival of Balb/C Mice Following Immunization with IT-1 PS¹

Immunized with (ug)	No. Alive Total	Percent Survivors	Challenge Dose
50	8/10	80	1.2×10^8
5	0/5	0	"
1	0/5	0	"
0	0/5	0	"

1 Mice given indicated amount of antigen in 0.1 ml saline followed on day 7 by challenge with live IT-1 P. aeruginosa in 0.1 ml saline.

2 one LD₁₀₀ equal to 1.2×10^8 live organisms

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